(For Laboratory Use Only)
ATS Labs Project # C15-9-13-13

PROTOCOL

AOAC Tuberculocidal Activity of Disinfectants

Test Organism:

Mycobacterium bovis – BCG

PROTOCOL NUMBER

DAC02082613.TB.1

PREPARED FOR

KIK International Inc.
909 Magnolia Ave
Auburndale, FL 33823

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PREPARED BY

Anne Stemper, B.S.
Senior Microbiologist

DATE

August 26, 2013

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AOAC Tuberculocidal Activity of Disinfectants

SPONSOR: KIK International Inc.
909 Magnolia Ave
Auburndale, FL 33823

TEST FACILITY: ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PURPOSE

The purpose of this assay is to evaluate the tuberculocidal effectiveness of a product against *Mycobacterium bovis* - BCG following the AOAC Tuberculocidal Activity Test. This method is in compliance with the requirements of the U.S. Environmental Protection Agency (EPA).

TEST SUBSTANCE CHARACTERIZATION

Test substance characterization as to content, stability, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor. The test substance shall be characterized by the Sponsor prior to the experimental start date of this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs.

SCHEDULING AND DISCLAIMER OF WARRANTY

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is September 5, 2013. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of October 2, 2013. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, due to failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test.

Neither the name of ATS Labs nor any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the regulatory agencies of its submission concerning report format, pagination, etc. To prevent rejection, the Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

Regulatory agencies require that a specific claim for a test substance intended for use on hard surfaces be supported by appropriate scientific data demonstrating the efficacy of the test substance against the claimed microorganism. This is accomplished by treating the target microorganism with the test substance under conditions, in the laboratory, which simulate as closely as possible the actual conditions under which the test substance is designed to be used. For test substances intended for use on hard surfaces (dry, inanimate environmental surfaces), a carrier method is used in the generation of the supporting data. The experimental design in this protocol meets these requirements.

TEST PRINCIPLE

A film of mycobacterial cells dried on porcelain penicylinder carriers is exposed to the test substance for a specified exposure time. After exposure, the carriers are transferred to vessels containing neutralizing subculture media and assayed for survivors. Appropriate culture purity, sterility, initial suspension, viability, neutralization confirmation and carrier population controls are performed. The current version of Standard Operating Procedure *CGT-4360 reflects the methods which shall be used in this study.*

TEST METHOD

Test Organism	Growth Medium	Incubation Parameters
Mycobacterium bovis (BCG)	Modified Proskauer-Beck Broth (MPB)	35-37°C, aerobic

The test organism to be used in this study was obtained from Organon Teknika, Durham, NC.

Carriers

Porcelain penicylinders (O.D. 8mm ± 1, I.D. 6mm ± 1, length 10mm ± 1) will be washed with 1-5% Triton X-100 and rinsed with water at least four times or until no soap residue is present. Following washing, each carrier will be macroscopically inspected for scratches, chips and cracks. Any carriers with visible defects will not be used. Carriers will be placed in a vessel and sterilized for ≥ 2 hours in ≥ 180°C air oven.

Preparation of Test Organism

A stock culture of the test organism, *Mycobacterium bovis* – BCG, is maintained on 7H11 agar medium. From the stock culture, the organism will be transferred into 20 mL tubes of Modified Proskauer-Beck Broth and incubated for 21±2 days at 35-37°C. Slanting the culture tubes is recommended. Following incubation, transfer the test culture to a sterile tissue grinder containing 1.0 mL of 0.85% saline + 0.1% tween 80 using a transfer loop. If necessary, a pipette may be used to transfer culture. Macerate the culture to break up large clumps or aggregates of the test organism. Add 9 mL Modified Proskauer-Beck broth to the culture and transfer the suspension from the tissue grinder to a sterile test tube. Allow the suspension to settle for approximately 10–15 minutes. Remove the upper portion of culture, leaving behind any debris or clumps, and transfer to a sterile vessel. This culture will be standardized to 20±1% Transmittance (%T) at 650 nm. Multiple cultures may be harvested, prepared and pooled as necessary. Modified Proskauer-Beck broth will be used for any applicable culture dilutions.

An organic soil load may be added to the test culture per Sponsor's request.

Contamination of Carriers

The penicylinders will be immersed for 15±1 minutes in the prepared culture at a ratio of one carrier per one mL culture (or sufficient volume of culture to cover the carriers). A minimum of six inoculated carriers will be needed for controls. Following inoculation, remove the carriers and shake carrier against the side of the inoculation vessel to remove excess culture. The carriers will then be dried in a sterile Petri dish matted with 2 pieces of Whatman #2 filter paper at 35-37°C for 30±2 minutes. Place no more than 12 carriers in a single Petri dish. The drying conditions (temperature and humidity) will be appropriate for the test organism for the purpose of obtaining maximum survival following drying. The actual drying conditions will be clearly documented. The carriers will be used in the test procedure within 2 hours following drying.

Preparation of Test Substance

The test substance(s) to be assayed will be used as directed by the Sponsor. If a dilution of the test substance is requested by the Sponsor, the diluted test substance(s) shall be used within three hours of preparation. Ten (10) mL of the test substance at its use-dilution will be aliquotted into the required number of sterile 25 x 150 mm or 25 x 100 mm tubes. The tubes will be placed into a waterbath at the specified exposure temperature, and allowed to equilibrate for ≥10 minutes prior to testing.

Exposure Conditions

Each contaminated and dried carrier is placed into a separate tube containing 10 mL of the test substance at its use-dilution for the desired exposure time and temperature.

Test System Recovery
Following the Sponsor specified exposure time, each medicated carrier will be transferred by wire hook at staggered intervals to 10 mL of neutralizer draining excess disinfectant from the carrier prior to transfer. Shake the tube containing the carrier in neutralizer and transfer the carrier to a tube containing 20 mL of Modified Proskauer-Beck Broth. Within approximately 30 minutes of neutralization, transfer a 2.0 mL aliquot of the neutralizer from each tube to individual tubes containing 20 mL of Middlebrook 7H9 Broth and 20 mL of Kirchner's Medium. Shake each subculture tube thoroughly.

Incubation and Observation
All plates will be incubated for 17-21 days at 35-37°C. The plates will be incubated in an appropriate manner to prevent desiccation. Following incubation, the organism plates will be visually examined for growth and enumerated if applicable. Plates may be stored at 2-8°C for up to 3 days prior to enumeration.

All subculture broths will be incubated at 35-37°C under aerobic conditions. The subcultures will be visually examined for growth following a 30 and 60 day incubation period or the first working day thereafter. If all test subcultures demonstrate lack of growth of the test organism, the subcultures will be incubated an additional 30 days and re-examined.

If the test subcultures demonstrate the presence of the test organism following the 60-day reading, the study will be completed. Representative test subcultures demonstrating growth will be stained using an AFB fluorescent stain to confirm identity of test organism.

STUDY CONTROLS

Purity Control
A "streak plate for isolation" will be performed on the organism culture and following incubation examined in order to confirm the presence of a pure culture. The acceptance criterion for this study control is growth of a pure culture demonstrating colony morphology typical of the test organism.

Organic Soil Load Sterility Control
If used in testing, the serum used for soil load will be cultured, incubated, and visually examined for lack of growth. The acceptance criterion for this study control is lack of growth.

Carrier Sterility Control
A representative uninoculated carrier will be added to a tube of Modified Proskauer-Beck Broth and incubated. The acceptance criterion is lack of growth.

Neutralizer and Subculture Medium Sterility Control
Two (2.0) mL aliquots of the neutralizer will be added to individual 20 mL tubes of Modified Proskauer-Beck Broth, Middlebrook 7H9 Broth and Kirchner's Medium. The subculture broths will be incubated and visually examined for growth. The acceptance criterion is lack of growth.

Viability Control
A representative inoculated carrier will be added to individual vessels of Modified Proskauer-Beck Broth, Middlebrook 7H9 Broth and Kirchner's Medium. The subculture broths will be incubated and visually examined for growth. The acceptance criterion for this study control is growth in all three subculture media.

Initial Suspension Population Control
The initial suspension population of the test organism will be determined using the dilutions plated in duplicate for the neutralization confirmation control. This control is for informational purposes and therefore has no acceptance criterion.

Neutralization Confirmation Control – Neutralizer Effectiveness
The neutralization of the test substance will be confirmed by exposing sterile carriers to the test substance and transferring to 10 mL of neutralizer. The carriers will be subcultured to Modified Proskauer-Beck Broth, identically to the test procedure. Two (2.0) mL aliquots of the neutralizer will be transferred to individual vessels containing 20 mL of Middlebrook 7H9 Broth and Kirchner's Medium in a manner consistent with the test procedure. The subculture broths will be inoculated with a low level of test organism. Shake each subculture vessel. The use of 0.1 mL aliquots from the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions are recommended. A standardized spread plate procedure will be run concurrently in order to enumerate the average number of CFU actually added by plating identical aliquots in duplicate. The plates and broth vessels will be incubated and evaluated for growth. Only the most concentrated test substance and/or shortest exposure time is required to be tested in this control. The acceptance criterion for this study control is growth following inoculation with low levels (e.g. ≤100 CFU) of test organism in the vessel containing the carrier, minimally. This control may be performed prior to testing or concurrent with testing.

Neutralization Confirmation Control – Neutralizer Toxicity
In order to determine the potential for bacteriostasis associated with the neutralizer, sterile carriers will be transferred to 10 mL of neutralizer. The carriers will be subcultured to Modified Proskauer-Beck Broth, identically to the test procedure. Two (2.0) mL aliquots of the neutralizer will be transferred to individual vessels containing 20 mL of Middlebrook 7H9 Broth and Kirchner's Medium in a manner consistent with the test procedure. The subculture broths will be inoculated with a low level of test organism. Shake each subculture vessel following inoculation. The use of 0.1 mL aliquots from the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions are recommended. The broth vessels will be incubated and evaluated for growth. The acceptance criterion for this study control is growth following inoculation with low levels (e.g. ≤100 CFU) of test organism in the vessel containing the carrier, minimally. This control may be performed prior to testing or concurrent with testing.

Neutralization Confirmation Control – Positive Control/Media Quality Assessment
Duplicate untreated vessels of Modified Proskauer-Beck Broth, Middlebrook 7H9 Broth and Kirchner's Medium will be inoculated with a low level of test organism as a positive control for comparison purposes. Shake each subculture vessel following inoculation. The use of 0.1 mL aliquots from the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions are recommended. The positive control subcultures are included for comparison purposes and can be used for baseline media performance. The acceptance criterion for this study control is growth following inoculation with low levels (e.g. ≤100 CFU) of test organism in both vessels for all three media types. This control may be performed prior to testing or concurrent with testing.

Carrier Population Control
Three inoculated and dried carriers will be randomly selected for the analysis in this control. One carrier will be assayed prior to testing and two carriers will be analyzed following testing. Each carrier will be transferred to a tube containing 10 mL of subculture broth (e.g. MPB broth) and sonicated for approximately 10 minutes. After sonication, briefly vortex mix the each tube and prepare ten-fold serial dilutions. If serial dilutions are not performed and plated immediately following sonication, the vessels may be refrigerated at 2-8°C for up to two hours prior to dilution. Plate duplicate 0.1 mL aliquots of appropriate dilutions onto Middlebrook 7H11 agar. Following incubation, the resulting colonies will be enumerated and the CFU per carrier calculated. The individual CFU per carrier set results will be calculated, and the Log_{10} value of each carrier set determined. The average Log_{10} value per organism will be calculated. The acceptance criterion for this study control is a minimum average Log_{10} value of 4.0.

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PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM

ATS Labs maintains Standard Operating Procedures (SOPs) relative to efficacy testing studies. Efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including test organism strains for purposes of identification, receipt and use of chemical reagents. These procedures are designed to document each step of efficacy testing studies. Appropriate references to medium, batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each efficacy test is assigned a unique Project Number when the protocol for the study is initiated by the Study Director. This number is used for identification of the test subcultures, etc. during the course of the test. Test subcultures are also labeled with reference to the test organism, experimental start date, and test product. Microscopic and/or macroscopic evaluations of positive subcultures are performed in order to confirm the identity of the test organism. These measures are designed to document the identity of the test system.

METHOD FOR CONTROL OF BIAS: NA

STUDY ACCEPTANCE CRITERIA

Test Substance Performance Criteria

The U.S. EPA efficacy performance requirements for label claims state that the test substance must kill the microorganism on 10 out of the 10 inoculated carriers.

Control Acceptance Criteria

The study controls must perform according to the criteria detailed in the study controls description section. If any of the control acceptance criteria are not met, the test may be repeated under the same protocol number. In the event that test organism growth is confirmed in the test in combination with a viability or neutralization control failure, no retesting is necessary. Additionally, if the population control exceeds an average $\log_{10}$ value of 6.0 and the test substance fails to meet the performance criteria, the Sponsor may invalidate the study and repeat testing.

REPORT

The report will include, but not be limited to, identification of the test substance, date received, initiation and completion dates, identification of the bacterial strain used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by 40 CFR Part 160.185.

PROTOCOL CHANGES

If it becomes necessary to make changes in the approved protocol, the revision and reasons for changes will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

Standard operating procedures used in this study will be the correct effective revision at the time of the work. Any minor changes to SOPs (for this study) or methods used will be documented in the raw data and approved by the Study Director.

TEST SUBSTANCE RETENTION

It is the responsibility of the Sponsor to retain a sample of the test substance. All unused test substance will be discarded following study completion unless otherwise indicated by Sponsor.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation, and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of final study report.
7. Study-specific SOP deviations made during the study.

Facility Specific Documents

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

REFERENCES

1. Association of Official Analytical Chemists (AOAC), Official Method 965.12. Tuberculocidal Activity of Disinfectants. In Official Methods of Analysis of the AOAC, 2012 Edition.
2. Association of Official Analytical Chemists (AOAC), Official Method 960.09. Germicidal and Detergent Sanitizing Action of Disinfectants Method [Preparation of Synthetic Hard Water], In Official Methods of Analysis of the AOAC, 2013 Edition.
3. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2000: General Considerations for Uses of Antimicrobial Agents, September 4, 2012.
4. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2200: Disinfectants for Use on Hard Surfaces- Efficacy Data Recommendations, September 4, 2012.

DATA ANALYSIS

Calculation

Determine the CFU/Carrier set in the Carrier Population Control using all average counts between 0-300 CFU as follows:

$$\text{CFU/carrier} = \frac{[(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})] \times (\text{Volume of neutralizer})}{[10^{-x} + 10^{-y} + 10^{-z}] \times (\text{Volume plated}) \times (\#\text{ of carriers per set})}$$

where 10^{-x}, 10^{-y}, and 10^{-z} are example dilutions that may be used

$$\text{Average Log}_{10}\text{ Carrier Population Control} = \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \ldots \text{Log}_{10}X_N}{N}$$

Where: X equals CFU/carrier set
N equals number of control carrier sets

Determine the Initial Suspension as follows:

$$\text{CFU/mL} = \frac{[(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})]}{[10^{-x} + 10^{-y} + 10^{-z}] \times (\text{Volume plated})}$$

where 10^{-x}, 10^{-y}, and 10^{-z} are example dilutions that may be used

Statistical Analysis
None used.

STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Test Substance (Name and Batch or Lot Number - exactly as it should appear on final report):
PURE BRIGHT GERMICIDAL ULTRA BLEACH EPA REG. NO. 70271-13
LOTS 13231601MIFL01 AND 13226174SMIFL01

Expiration Date: JAN 2014

Product Description:
- ☐ Quaternary ammonia
- ☐ Iodophor
- ☒ Sodium hypochlorite
- ☐ Peracetic acid
- ☐ Peroxide
- ☐ Other_____

Test Substance Active Concentration (upon submission to ATS Labs): 6%

Neutralization/Subculture Broth:
- ☐ _____
- ☐ Horse Serum
- ☐ Horse Serum + 0.1% sodium thiosulfate
- ☒ ATS Labs' Discretion. By checking, the Sponsor authorizes ATS Labs, at their discretion, to perform neutralization confirmation assays at the Sponsor's expense prior to testing to determine the most appropriate neutralizer. (See Fee Schedule).

Storage Conditions
- ☒ Room Temperature
- ☐ 2-8°C
- ☐ Other_____

Hazards
- ☐ None known: Use Standard Precautions
- ☒ Material Safety Data Sheet, Attached for each product
- ☐ As Follows:_____

Product Preparation
- ☐ No dilution required, Use as received (RTU)
- ☑ *Dilution(s) to be tested:
  6,050-6,250 ppm defined as See protocol modifications + _____
  (example: 1 oz/gallon) (amount of test substance) (amount of diluent)
  - ☐ Deionized Water (Filter or Autoclave Sterilized)
  - ☑ Tap Water (Filter or Autoclave Sterilized)
  - ☐ AOAC Synthetic Hard Water: _____PPM
  - ☐ Other _____

Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Test Organism: *Mycobacterium bovis*-BCG

Carrier Number: 10 per batch

Exposure Time: 10 minutes **Exposure Temperature:** 20±1°C

Organic Soil Load:
- ☑ Minimum 5% Organic Soil Load (fetal bovine serum)
- ☐ No Organic Soil Load Required
- ☐ Other _____

TEST SUBSTANCE SHIPMENT STATUS

- ☐ Has been used in one or more previous studies at ATS Labs.
- ☐ Has been shipped to ATS Labs (but has not been used in a previous study).
  Date shipped to ATS Labs:_____ Sent via *overnight* delivery? ☐ Yes ☐ No
- ☑ Will be shipped to ATS Labs.
  Date of expected receipt at ATS Labs: AUG. 30, 2013
- ☐ Sender (if other than Sponsor):_____

COMPLIANCE

Study to be performed under EPA Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures.
☑ Yes
☐ No (Non-GLP Study)

PROTOCOL MODIFICATIONS

- ☐ Approved without modification
- ☑ Approved with modification

Per Sponsor's request, the test substance concentrate will be titrated on the day of testing for available chlorine following ATS Labs SOP CGT-0090. The result of this titration will be used to prepare an available chlorine concentration of between 6,050 – 6,250 ppm for use in testing. The diluted test concentration will also be titrated to confirm the concentration. If the in-use concentration is out of range, the test substance will be re-prepared or adjusted and titrated prior to efficacy testing

PROTOCOL ATTACHMENTS

Supplemental Information Form Attached - ☐ Yes ☑ No

APPROVAL SIGNATURES

SPONSOR:

NAME: Mr. Justin Lowe TITLE: REGIONAL QA MGR.

SIGNATURE: Justin Lowe DATE: 8/28/2013

PHONE: (863) 551 - 3006 FAX:_____ EMAIL: jlowe@kikcorp.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study: ☐ See Attached

CRISTINA GRIFFIN, DELTA ANALYTICAL CORP. (CGRIFFIN@DELTA-AC.COM)

ATS Labs:

NAME: Matthew Sathe
Study Director

SIGNATURE: Matth Sath DATE: 9-4-13
Study Director